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N-Linked Protein Glycosylation Is Required for Full Competence in *Campylobacter jejuni* 81-176

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The recent sequencing of the virulence plasmid of *Campylobacter jejuni* 81-176 revealed the presence of genes homologous to type IV secretion systems (TFSS) that have subsequently been found in *Helicobacter pylori* and *Wolinella succinogenes*. Mutational analyses of some of these genes have implicated their involvement in intestinal epithelial cell invasion and natural competence. In this report, we demonstrate that one of these type IV secretion homologs, Cjp3/VirB10, is a glycoprotein. Treatment with various glycosidases and binding to soybean agglutinin indicated that the structure of the glycan present on VirB10 contains a terminal GalNAc, consistent with previous reports of N-linked glycans in *C. jejuni*. Site-directed mutagenesis of five putative N-linked glycosylation sites indicated that VirB10 is glycosylated at two sites, N32 and N97. Mutants in the N-linked general protein glycosylation (*pgl*) system of *C. jejuni* are significantly reduced in natural transformation, which is likely due, in part, to lack of glycosylation of VirB10. The natural transformation defect in a *virB10* mutant can be complemented in *trans* by using a plasmid expressing wild-type VirB10 or an N32A substitution but not by using a mutant expressing VirB10 with an N97A substitution. Taken together, these results suggest that glycosylation of VirB10 specifically at N97 is required for the function of the TFSS and for full competence in *C. jejuni* 81-176.

Campylobacter jejuni 81-176 is unique for a prokaryotic organism in that it has a general system of N-linked protein glycosylation (*pgl*), affecting a substantial number of periplasmic and surface proteins (29, 38). The structure of the N-linked glycan present on *C. jejuni* NCTC 11168 glycoproteins was recently found to be a heptasaccharide with a mass of 1,406 Da composed of GalNAc-α1,4-GalNAc-α1,4-(Glc-β1,3-)GalNAc-α1,4-GalNAc-α1,4-GalNAc-α1,3-bacillosamine (38). There are several genes in the *C. jejuni pgl* locus that have been shown to be involved in distinct steps in N-linked glycosylation (38). The addition of the glycan is dependent on the activity of the PglB protein, which is predicted to function as an oligosaccharide transferase (29, 32, 38) based on its homology to an oligosaccharide transferase subunit (STT3) of *Saccharomyces cerevisiae* (39). Other *pgl* genes (*pglF*, *pglE*, and *pglD*) have been proposed to be involved in synthesis of bacillosamine, a sugar that appears to be specific to this N-linked glycan (32, 38). Mutation of either *pglB* or *pglE* diminished the ability of 81-176 to invade INT407 cells and colonize the intestinal tracts of mice (28), reinforcing the importance of protein glycosylation to the pathogenesis of *C. jejuni*. However, the precise functional contribution of N-linked glycosylation to the pathogenesis of *C. jejuni* remains unclear.

C. jejuni strain 81-176 possesses two plasmids, one of which, pVir, is nonconjugative and affects both virulence and natural competence (2). Sequence analysis of this plasmid revealed the presence of eight genes with greatest homology to a type IV secretion system (TFSS) subsequently shown to be present in the ruminant commensal *Wolinella succinogenes* (1, 3). There is also significant homology to two TFSS found in *Helicobacter pylori*. These are the *com* system, which is responsible for natural transformation in *H. pylori*, and a more recently described TFSS of unknown function found in the *H. pylori* J99 plasticity zone (15, 16, 19). In contrast, the pVir TFSS shows much less homology to the well-characterized TFSS found on the *cag* pathogenicity island of *H. pylori* (7, 31). TFSS, which are present in a variety of plant and mammalian pathogens, are involved in the transfer of DNA, protein, or nucleoprotein complexes across bacterial membranes (8). The TFSS genes present on pVir have been proposed to encode proteins that form a functional secretion channel that appears to affect both intestinal epithelial cell invasion and natural competence (2, 3).

Herein we report that a putative structural component of the pVir TFSS, VirB10 (Cjp3) (3), is glycosylated by the *pgl* system at two asparagines residues and that lack of glycosylation at one site results in a competence defect comparable to that of the *virB10* mutant. Further, we demonstrate that *pgl* mutants exhibit a major defect in natural competence, suggesting that N-linked glycosylation is required for full competence in *C. jejuni* 81-176.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *C. jejuni* DB179 (81-176 cured of the second plasmid, pTet) has been described previously (2). All *C. jejuni* mutants used were constructed by insertional inactivation of the target gene by either an *aph3A* cassette (Km^r) (2) or insertion of a *cat* cassette using an EZ::TN transposon containing a campylobacter *cat* gene (Cm^r) (3). The 81-176 *pgl* mutants were Km^r insertions into *pglE* or *pglB* and have been described previously (29). The *virB8*::Cm and *virB9*::Cm mutations in pVir have been previously described (3). The pVir *virB10*::Km mutation (2) was reconstructed in DB179 to avoid any potential interaction with the conjugative TFSS system encoded by the pTet

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TABLE 1. Plasmids used

Plasmid	Description	Reference or source
pBTLPS	Entire *pgl* operon cloned in pACYC184 (Cm^r Tc^s)	32
pCE107/70	*C. jejuni*/*E. coli* expression vector; derivative of pRY107, a Km^r campylobacter shuttle vector, with σ^{70} promoter from Cj1291	37 and this work
pCE111/28	*C. jejuni* expression vector; derivative of pRY111, a Cm^r campylobacter shuttle vector, with σ^{28} promoter from *flaA*	37 and this work
pJL101	*virB10* cloned into pCE107/70	This work
pJL102	*virB10* cloned into pCE111/28	This work
pJL102/N32A	N32A substitution in *virB10* coding sequence in pJL102	This work
pJL102/N42A	N42A substitution in *virB10* coding sequence in pJL102	This work
pJL102/N97A	N97A substitution in *virB10* coding sequence in pJL102	This work
pJL102/N32A, N97A	N32A and N97A substitutions in *virB10* coding sequence in pJL102	This work
pCS101	pRY111, Cm^r campylobacter shuttle vector carrying *pglE* under control of *pglE* promoter	29

plasmid also resident in 81-176. All mutants were tested by PCR using primers that bracket the insertion point of the drug resistance gene to confirm a double crossover (2, 3). *C. jejuni* was grown at 37°C under microaerobic conditions on Mueller-Hinton (MH) agar (Difco). *Escherichia coli* DH5α transformed with the *C. jejuni pgl* genes present on pACYC184 (pBTLPS) has been described previously (32). *E. coli* strains were grown on Luria agar. *E. coli* DH5α was used as the host strain for cloning experiments, and DH5α containing pRK212.1 was used as the donor in conjugation experiments (10). *E. coli* ER2566 (New England Biolabs, Beverly, Mass.) was used as the host strain for protein expression experiments. Antibiotics were added when appropriate to the following concentrations: 100 μg of ampicillin per ml, 20 μg of chloramphenicol per ml, 25 μg of kanamycin per ml, 20 μg of streptomycin per ml, 20 μg of tetracycline per ml, and 10 μg of trimethoprim per ml. Plasmids used are listed in Table 1.

Construction of campylobacter expression vectors. The region upstream of Cj1291, designated *accB*, a putative biotin carboxyl carrier protein of acetyl-coenzyme A carboxylase (11, 27) containing a putative σ^{70} promoter, was PCR amplified with HF2 DNA polymerase (Clontech, Palo Alto, Calif.) with the following primers: 5′-CGGGATCCCGAAAATTCTCCTACAAAATTTAAGAAC-3′ and 5′-GCTCTAGAGCTTTTAACCTTTTAATATTAGTAATTTTTT-3′. These primers introduced BamHI and XbaI sites bracketing the promoter region of Cj1291. The PCR product was digested with BamHI and XbaI and was cloned into BamHI-XbaI digested pRY107, a kanamycin-resistant shuttle vector (37), to generate pCE107/70. The region upstream of the *flaA* gene containing the σ^{28} promoter was PCR amplified from 81-176 by using HF2 DNA polymerase (Clontech) with the following primers: 5′-GCTCTAGAGCGTAAAATTGAAGATGAAAGAGAG-3′ and 5′-CGGGATCCCGTTTTAAATCCTTTTAAATAATTTC-3′. These primers introduced XbaI and BamHI sites, respectively. The PCR product was digested with XbaI and BamHI (New England Biolabs) and cloned into XbaI-BamHI-digested pRY111, a chloramphenicol-resistant campylobacter shuttle plasmid (37), to generate pCE111/28.

Complementation in *trans* of the *virB10* mutation. PCR amplification was used to amplify *virB10* (*cjp3*) from the pVir plasmid by using HF2 DNA polymerase (Clontech). The primers to amplify *cjp3*/*virB10* were JCL 075 (5′-CGCGGATCCATGAAAAAATCCTTTTTAAGCC-3′) and JCL 076 (5′-GGCTGCAGTTAATTATCTTGGAAATATTGG-3′), which introduced BamHI and PstI sites (5′ and 3′, respectively) flanking the *virB10* coding sequence. The amplicon was digested with BamHI and PstI and was cloned into the BamHI and PstI sites of pCE107/70 or pCE111/28 to create pJL101 or pJL102, respectively. The pJL102 construct and mutant derivatives were mobilized from *E. coli* DH5α containing pRK212.1 into *C. jejuni* DB179 *virB10*::Km cells. Transconjugants were selected on MH agar containing kanamycin, chloramphenicol, and trimethoprim. Plasmid pJL101 was transformed into *E. coli* DH5α with or without pBTLPS (32).

Site-directed mutagenesis of *virB10*. Mutation of five of the six predicted N-linked glycosylation sites of VirB10 was carried out using the Quickchange site-directed mutagenesis kit (Stratagene, La Jolla, Calif.). Mutations were confirmed by sequencing with a Big Dye Terminator sequencing kit (Applied Biosystems, Foster City, Calif.) on an ABI Prism 3100 genetic analyzer (Applied Biosystems). Oligonucleotides used are listed in Table 2. The construction of the single mutants was carried out with pJL102 as a template, and pJL102/N32A was used as the template to construct the double mutant, pJL102/N32, N97A.

Cloning and expression of VirB8, VirB9, and VirB10. The genes encoding VirB8 (Cjp1), VirB9 (Cjp2), and VirB10 (Cjp3) were fused to intein in the expression vector pTYB12 (New England Biolabs) by the following procedure. First, the DNA fragments were generated by PCR amplification using HF2 DNA polymerase (Clontech). The primers, which introduced SpeI and EcoRI sites, were *cjp1*-F (5′-GACTAGTGGAATGAGTAATAATACTATTGT-3′), *cjp1*-R (5′-GCGAATTCGTTACTTCGCTCCTTTCGTTTG-3′), *cjp2*-F (5′-GACTAGTGGAGACAACATACAAATTCAAGATGTTCC-3′), *cjp2*-R (5′-GCGAATTCGTCATTTCTTAGCCTT-3′), *cjp3*-F (5′-GACTAGTGGACAAACAAGCGAAGAAAATGTATC-3′), and *cjp3*-R (5′-GGAATTCTTAATTATCTTGGAAATATTGGATCAATA-3′). The PCR products were digested with SpeI and EcoRI and were cloned into pTYB12 (New England Biolabs). The pTYB12-Cjp1 clone contains an N-terminal intein fusion containing residues 52 to 225 of Cjp1. The pTYB12-Cjp2 and pTYB12-Cjp3 clones contain residues 22 to 356 and 28 to 378, respectively. *E. coli* ER2566 containing these constructs was grown overnight in Luria-Bertani medium at 37°C. The cells were diluted 1:100 in fresh medium containing ampicillin, and 1-liter cultures were grown to an optical density at 600 nm of approximately 0.5. Isopropyl-β-D-galactoside was added to

TABLE 2. Oligonucleotides used for the site-directed mutagenesis of *C. jejuni virB10*

Name	Sequence
5′ *virB10* N32A	5′ GCAGAAGATATATTTGATCAAACAAGCGAAGAAGCTGTATCTAAAAATATATCTAAAAAAGACAATCAAAGC 3′
3′ *virB10* N32A	5′ GCTTTGATTGTCTTTTTTAGATATATTTTTAGATACAGCTTCTTCGCTTGTTTGATCAAATATATCTTCTGC 3′
5′ *virB10* N42A	5′ CGAAGAAAATGTATCTAAAAATATATCTAAAAAAGACGCTCAAAGCCAAAATTTGCTTAACAAAGATTTAG 3′
3′ *virB10* N42A	5′ CTAAATCTTTGTTAAGCAAATTTTGGCTTTGAGCGTCTTTTTTAGATATATTTTTAGATACATTTTCTTCG 3′
5′ *virB10* N97A	5′ CATAGTGAAGAAAAACCTAAAAAAGAAGAAGATAATGCTATTACTAAGTTAGCAAAAATTGAAGAAAAAAAGCAAGAAC 3′
3′ *virB10* N97A	5′ GTTCTTGCTTTTTTTCTTCAATTTTTGCTAACTTAGTAATAGCATTATCTTCTTCTTTTTTAGGTTTTTCTTCACTATG 3′
5′ *virB10* N126A	5′ CAGCAAATTGCAAAAGAAATTCATCAAGATGCTATTAGTTCTCAAGAAAGAAAAATC 3′
3′ *virB10* N126A	5′ GATTTTTCTTTCTTGAGAACTAATAGCATCTTGATGAATTTCTTTTGCAATTTGCTG 3′
5′ *virB10* N156A	5′ CAACACGCAAATTTATTTTCAAGAAGCTTCAAAATACGGCGTTGATGGTTTTTC 3′
3′ *virB10* N156A	5′ GAAAAACCATCAACGCCGTATTTTGAAGCTTCTTGAAAATAAATTTGCGTGTTG 3′

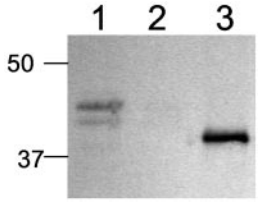

FIG. 1. Discrepancy in mass of VirB10 expressed in *C. jejuni* and *E. coli*. Immunoblot of recombinant VirB10 isolated from *E. coli* and glycine extracts from *C. jejuni* DB179 and isogenic *virB10* mutant. Protein samples were separated on a 10% acrylamide gel. Blots were incubated with an anti-VirB10 antiserum at 1:50,000 dilution. Lane 1, DB179; lane 2, DB179 (pVir/*virB10*::Km); lane 3, recombinant VirB10.

a final concentration of 0.5 mM, and cultures were grown overnight at 16°C. Bacteria were pelleted by centrifugation at 5,000 × *g* for 10 min. Cell pellets were stored frozen at −20°C. Affinity chromatography was carried out using the IMPACT-CN protein purification system according to the manufacturer's recommendations (New England Biolabs).

Generation of polyclonal antisera. Protein samples were sent for injection into New Zealand White Rabbits at Harlan Bioproducts (Indianapolis, Ind.). Following the manufacturer's immunization protocol, polyclonal antisera were obtained and used at the indicated dilutions.

Electrophoresis and immunoblotting. *Campylobacter* spp. and *E. coli* whole cells were resuspended in 1× sodium dodecyl sulfate-polyacrylamide electrophoresis (SDS-PAGE) sample buffer to a final protein concentration of 10 μg/μl. Protein samples were aliquoted and resuspended in an equal volume of 2× SDS-PAGE sample buffer. Samples were boiled and loaded onto 10% acrylamide gels. Proteins were separated by SDS-PAGE (21) and were detected by staining with Coomassie brilliant blue G250 or, after transfer to nitrocellulose, Western blot analysis using the indicated rabbit antisera. The secondary antibody was goat anti-rabbit antiserum conjugated to alkaline phosphatase (Caltag, Burlingame, Calif.) used at a 1:5,000 dilution.

SBA affinity columns. Glycine extracts were prepared by resuspending a loopful of campylobacter organisms in 0.2 M glycine-HCl, pH 2.2, and placing it on ice for 10 min. Samples were centrifuged at 16,000 × *g* and suspended in an equal volume of 2× SDS-PAGE sample buffer. Large-scale glycine extracts were prepared with 100 ml of *C. jejuni* grown in biphasic culture as previously described (24). Prepared glycine extracts were incubated with 2 ml of soybean agglutinin (SBA) agarose (Vector Labs, Burlingame, Calif.) at 4°C. Following the collection of flowthrough fractions, the column was washed in 20 ml of column buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.1 mM $CaCl_2$, and 0.01 mM $MnCl_2$) and wash fractions were collected. Proteins were eluted from the column by washing with 3 bed volumes of column buffer containing 0.2 M galactose.

Enzymatic deglycosylation. Neutralized glycine extracts of *C. jejuni* were treated with α-*N*-acetyl-galactosaminidase or β-*N*-acetylhexosaminidase (New England Biolabs) according to the manufacturer's recommendations. After the addition of an equal volume of 2× SDS-PAGE loading buffer, samples were boiled and loaded onto a 10% acrylamide gel.

Natural transformation of *C. jejuni*. The biphasic natural transformation procedure was used as previously described (33). *C. jejuni* strains were grown overnight on plates and were resuspended in MH broth to an optical density at 600 nm of 1.0. Aliquots of 250 μl of each strain were grown for an additional 2 h at 37°C in biphasic culture tubes (13). DNA (500 ng) from a streptomycin-resistant mutant of 81-176 (13) was added to cultures, and incubation continued for 4 h at 37°C. Cultures were serially diluted and plated in duplicate to MH agar containing streptomycin. The results were expressed as the number of transformants per microgram of Str^r DNA. Negative controls were treated identically without the addition of DNA.

RESULTS

Discrepancy between predicted and observed mass of VirB10. As shown in Fig. 1, lane 1, VirB10 expressed in *C. jejuni* DB179 presents two bands: a major band with an apparent molecular mass of approximately 43 kDa and a minor band with an apparent molecular mass of 41.5 kDa. The major 43-kDa band is substantially larger than that of the recombinant form of VirB10 expressed in *E. coli* (40.1 kDa) (lane 3) or the predicted mass of the mature VirB10 lacking its signal peptide (40.5 kDa). The significant discrepancy in mass between the recombinant protein expressed in *E. coli* and native forms of VirB10 suggested that VirB10 may be glycosylated in *C. jejuni*.

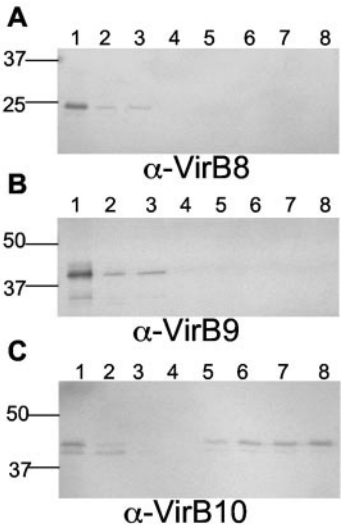

FIG. 2. VirB10 possesses affinity for SBA. Neutralized glycine extracts of DB179 were subjected to column chromatography with a SBA-agarose column. Extract, flowthrough, wash, and elution fractions were subjected to SDS-PAGE, blotted onto membranes, and incubated with either anti-VirB8 (A), anti-VirB9 (B), or anti-VirB10 antisera (C) at a 1:50,000 dilution. Lane 1, glycine extract from DB179; lane 2, column flowthrough fraction; lane 3, wash fraction; lanes 4 to 8, elution fractions.

VirB10 possesses affinity for SBA. Glycine extracts of DB179 contained VirB8, -9, and -10, suggesting a periplasmic or surface localization for these proteins (Fig. 2A to C, lanes 1). Glycine extracts were passed through an SBA-agarose column to bind N-linked glycoproteins containing a terminal GalNAc (23). Western blot analysis of elution fractions using antiserum against whole cells of 81-176 revealed the presence of multiple proteins, indicating that enrichment of glycoproteins had occurred (data not shown), consistent with Linton et al. (23). When blotted with anti-VirB10 antiserum, a major band corresponding to the native molecular mass (43 kDa) of VirB10 was detected in the elution fractions (Fig. 2C, lanes 5 to 8). The lower 41.5-kDa form of VirB10 also bound to and eluted from the column, but it did so in smaller amounts than the 43-kDa form. In contrast, neither VirB8 nor VirB9 was detected in the elution fractions that used antisera against recombinant forms of these proteins (Fig. 2A and B).

VirB10 susceptibility to glycosidases. Glycine-extracted proteins of *C. jejuni* DB179 were digested with glycosidases specific for HexNAc. After treatment with α-*N*-acetylgalactosaminidase, which cleaves the internal α1,3-linked *N*-acetylgalactosamine residue from the rest of the glycan, VirB10 mobility decreased on SDS-PAGE gels (Fig. 3, lane 2). Treatment with β-*N*-acetylhexosaminidase, which cleaves terminal β1-, β2-, β3-, β4-, and β6-linked GalNAc and *N*-acetylglucosamine residues, resulted in no discernible difference in mass (Fig. 3, lane 3). These data indicate that the glycan present on VirB10 contains an α1,3-linked Gal-

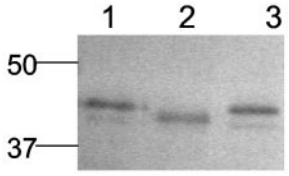

FIG. 3. VirB10 is susceptible to treatment with *N*-acetylgalactosaminidase. Neutralized glycine extracts of *C. jejuni* DB179 were untreated (lane 1), treated with *N*-acetylgalactosaminidase (lane 2), or treated with β-*N*-acetylhexosaminidase (lane 3). Samples were separated on a 10% acrylamide gel, blotted onto membranes, and incubated with anti-VirB10 antiserum at a 1:50,000 dilution.

NAc, consistent with the structure of the campylobacter N-linked glycan previously reported (38).

Restoration of wild-type mobility of VirB10 in *E. coli* expressing the *pgl* system. Recently it was demonstrated that the general protein glycosylation system of *C. jejuni* could be functionally reconstituted in *E. coli* (32). We took advantage of this information to provide genetic evidence that VirB10 is a glycoprotein. When VirB10 was expressed in *trans* from pJL101 (a *C. jejuni*/*E. coli* Km^r expression vector; see Table 1 and Material and Methods) in *E. coli* DH5α in the absence of the *pgl* system, two bands were visible (Fig. 4, lane 3). The major band corresponded to the mass of recombinant, unglycosylated VirB10 (Fig. 4, lane 1) lacking a signal peptide (40.5 kDa). The minor band in these whole-cell extracts, of approximately 41.5 kDa, likely represents VirB10 without its leader sequence removed (see below). In DH5α containing both pBTLPS, carrying the intact *pgl* operon (32), and pJL101, bands of similar apparent mass were observed (Fig. 4, lane 5) as well as an additional band that corresponded to the mass of the glycosylated form of VirB10 expressed in DB179 (Fig. 4, lane 6). When a clarified whole-cell extract from DH5α(pBTLPS, pJL101) was subjected to column chromatography using the SBA-agarose column, the major band detected in the elution fractions (Fig. 4, lane 7) corresponded in mass to that of glycosylated VirB10 expressed in DB179 (lane 6); there was also a minor band that had the same apparent mass as the middle band shown in lane 5 (see below). When a lysate from DH5α containing pJL101 but not pBTLPS was passed over SBA agarose, no VirB10 could be detected in the elution fractions by immunoblot (data not shown).

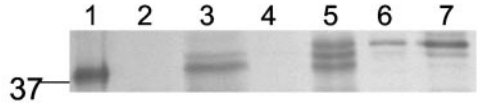

FIG. 4. Analysis of VirB10 expressed in *E. coli* DH5α in the presence or absence of the *C. jejuni pgl* system. Whole-cell extracts of *E. coli* were prepared and separated on a 10% acrylamide gel, blotted, and immunodetected with VirB10 antisera at 1:100,000 dilution. Lane 1, purified recombinant VirB10 from *E. coli*; lane 2, *E. coli* DH5α(pCE107/70), the vector-only control; lane 3, *E. coli* DH5α(pJL101), expressing *virB10* in the absence of the *pgl* system; lane 4, *E. coli* DH5α(pBTLPS) containing the *pgl* genes cloned into pACYC184 (32); lane 5, *E. coli* DH5α (pBTLPS, pJL101), expressing *virB10* in the presence of the *pgl* system; lane 6, control of a glycine extract from *C. jejuni* DB179; lane 7, elution fraction from SBA column of lysates from *E. coli* DH5α(pBTLPS, pJL101).

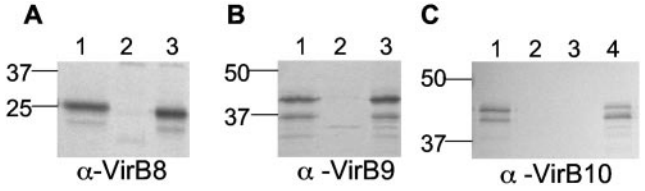

FIG. 5. VirB10 is absent in the periplasm of mutants defective in the general protein glycosylation pathway of *C. jejuni*. Glycine extracts of *C. jejuni* DB179 and mutants were separated on a 10% acrylamide gel and were blotted, and membranes were probed with either VirB8 (A), VirB9 (B), or VirB10 (C) antisera at a 1:50,000 dilution. Lanes 1, *C. jejuni* DB179; lanes 2, 81-176 *virB8*::Cm (A), 81-176 *virB9*::Cm (B), or DB179 *virB10*::Km (C); lanes 3, 81-176 *pglE*::Km; lane 4 of panel C, 81-176 *pglE*::Km (pCS101) (29).

VirB10 is not detected in *pglB* or *pglE* mutants. Western blot analysis of glycine extracts of the *pglB*::Km mutant (data not shown) or the *pglE*::Km mutant (29) failed to detect any VirB10 (Fig. 5C, lane 3). When the *pglE* mutation was complemented in *trans* with plasmid pCS101 (29), VirB10 was detected in the glycine extract (Fig. 5C, lane 4). Some expression of what appeared to be unglycosylated VirB10 was detected in whole cells of *pgl* mutants (data not shown). No detectable differences in expression patterns of VirB8 and VirB9 were observed between the wild-type and the *pglE* mutant (Fig. 5A and B).

Site-directed mutagenesis. VirB10 is predicted to contain six potential N-linked glycosylation sites (Asn-X-Ser/Thr). A series of site-directed mutagenesis experiments was done on plasmid pJL102, expressing *virB10* under control of the *flaA* σ^{28} promoter, to generate asparagine-to-alanine substitutions in five of these six possible glycosylation sites. Figure 6 shows a Western blot of glycine-extracted proteins of the DB179 *virB10*::Km mutant complemented in *trans* with selected mutated derivatives of pJL102. Alanine substitution of N32 of VirB10 (Fig. 6, lane 4) resulted in a decrease in mass such that the VirB10 band migrated at a position similar to that of the minor 41.5-kDa band seen in glycine extracts of DB179 (Fig. 6, lane 1). Mutation of N97 of VirB10 resulted in the presence of two equally intense bands (lane 5). The first band corresponded to the minor 41.5-kDa form of VirB10 detected in glycine extracts of DB179 (lane 1) and to that seen in N32A mutant (lane 4). The lower band migrated in parallel to the recombinant unglycosylated form of the protein expressed in *E. coli* (Fig. 6, lane 7). When both N32 and N97 were mutated in the same plasmid (lane 6), only one band was detected that was of the same apparent mass as the recombinant, nonglycosylated form of VirB10 (lane 7). Mutation of three other pu-

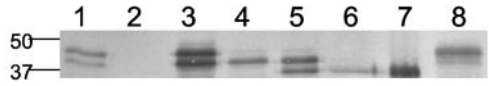

FIG. 6. VirB10 contains two N-linked glycosylation sites. Immunoblot of glycine extracted proteins of *C. jejuni* DB179 and mutants. Lane 1, DB179; lane 2, DB179 *virB10*::Km; lane3, DB179 *virB10*::Km (pJL102); lane 4, DB179 *virB10*::Km (pJL102N32A); lane 5, DB179 *virB10*::Km (pJL102N97A); lane 6, DB179 *virB10*::Km (pJL102N32A,N97A); lane 7, recombinant VirB10; lane 8, DB179 *virB10*::Km (pJL102N42A). Anti-VirB10 antiserum was used at a 1:50,000 dilution.

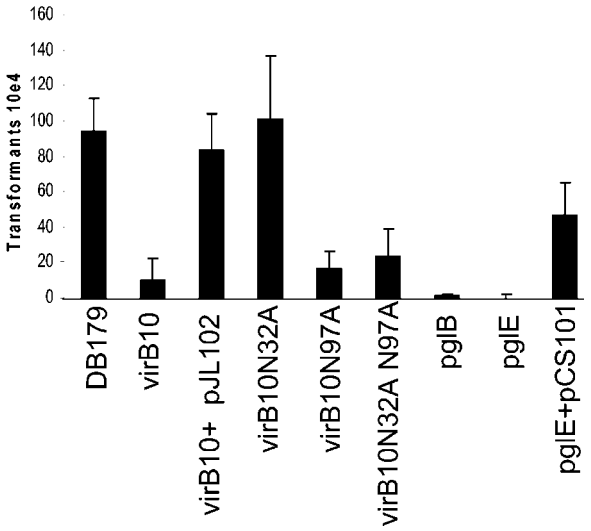

FIG. 7. Contribution of glycosylation to natural transformation. DNA (500 ng) from a streptomycin-resistant mutant of 81-176 was used to transform *C. jejuni* strains. Results are expressed as the total number of transformants per microgram of DNA and represent the means and standard deviations of at least three independent experiments. There was no difference in transformation ability of 81-176 and DB179 (data not shown).

tative N-linked glycosylation sites of VirB10 (N42, N126, and N156) resulted in no discernible difference in mass; representative results for N42A are shown in lane 8. These results suggest the presence of two glycosylation sites within VirB10: N32 and N97.

Effects of glycosylation on natural competence. It has previously been reported that mutation of *virB10* resulted in modest reductions in natural competence (2, 35). Because VirB10 is a glycoprotein, *pgl* mutants of 81-176 were tested for natural competence by using a chromosomal Str^r marker (13). Figure 7 demonstrates that a mutation in either *pglB* or *pglE* resulted in a significant decrease in the number of transformants. *C. jejuni* DB179 produced an average of 9.3×10^4 transformants per μg of Str^r DNA, which translates into efficiencies of 2.35×10^{-4} per μg/cell, within previously reported ranges (33, 35, 36). The *pglB* and *pglE* mutants transformed at frequencies of approximately 1.0×10^{-8}, an efficiency that is 10,000-fold lower than that of the wild type. When the *pglE* mutation was complemented in *trans* with pCS101, an increase in the level of competence was observed. The lack of complete complementation likely reflects instability of the plasmid, a phenomenon that has been observed with some genes in *trans* and with the complementation of other competence genes in *C. jejuni* (35 and P. Guerry, unpublished data). The *virB10* mutant was transformed as previously reported (2, 35) at a frequency of 2.3×10^{-5}, or approximately 10-fold lower than the frequency of the wild-type strain. The defect in transformation frequency observed in the *virB10* mutant was complemented in *trans* with pJL102 and restored it to wild-type levels. Wild-type levels of competence were exhibited in the mutant containing pJL102/N32A. However, pJL102/N97A failed to complement the *virB10* mutant, as did pJL102/N32A, N97A, which encodes a double mutation. These results suggest that glycosylation of VirB10 at N97, but not at N32, is essential for wild-type levels of competence in *C. jejuni* 81-176.

DISCUSSION

Evidence suggests that the glycosylation of bacterial proteins may contribute to a variety of cellular and pathogenic processes. Reduction in bacterial adherence and invasion was demonstrated in several organisms when glycosylation was prevented (5, 12, 20, 22, 26, 29). Other work has implicated protein glycosylation in antigenic variation, protection from proteolytic cleavage, and solubility (14, 17, 18, 25). The flagella of *C. jejuni* 81-176 are extensively modified with O-linked pseudaminic acid residues and derivatives (30). In the absence of any of these modifications, flagella filaments do not assemble, rendering the bacteria nonmotile and thus nonvirulent (11). Additionally, mutants in the *C. jejuni pgl* system have a reduced capacity to invade INT407 cells and a deficiency in their ability to colonize the intestinal tracts of mice (28).

The findings reported here demonstrate that the *C. jejuni* 81-176 pVir TFSS protein, VirB10, is glycosylated at two sites, N32 and N97. Thus, the two forms of VirB10 observed in wild-type DB179, both of which bound to the SBA-lectin column, represent mono- and diglycosylated forms. The minor band seen in *E. coli* whole cells containing pJL101 (Fig. 4, lane 3) most likely represents unprocessed VirB10. The small difference in mass (906 Da) between the VirB10 signal peptide and the N-linked glycan was not resolved in SDS-PAGE. Glycosylation of VirB10 at N97, but not N32, was essential for wild-type levels of competence. The predominant modification site appears to be N97, because the unmodified form of VirB10 (40.5 kDa) was present in glycine extracts of the VirB10 N97A mutant but not the VirB10 N32A mutant (Fig. 6). This suggests that the N97 site may be in a more favorable context for glycosylation than the N32A site, perhaps due to increased surface exposure.

The original phenotype described for *pgl* mutants was loss of immunoreactivity with a variety of antisera made against *C. jejuni* (29). This was interpreted as being due to the immunodominance of the glycan on proteins that were expressed at low levels. However, here we have reported that a glycosylated protein appeared to lose reactivity in a *pgl* mutant background with antiserum generated against a recombinant, unglycosylated form of the same protein. This would suggest that in the absence of glycosylation VirB10 either was not transported to the periplasm or, upon transport, was unable to interact with the other components of the TFS apparatus and was rapidly degraded. However, the VirB10 N32A, N97A mutant protein was detected in glycine extracts when overexpressed in *trans* from the *flaA* σ^{28} promoter, which is approximately 10-fold stronger than the native *virB10* promoter (P. Guerry, unpublished). This would suggest that the lack of detection of VirB10 in the *pgl* mutants reflects instability of the nonglycosylated protein, perhaps a result of an inability to interact with other TFSS proteins.

The six sites of potential glycosylation of the VirB10 homolog encoded by pVir are in contrast to the one or two putative glycosylation sites present in the *H. pylori* and *Agrobacterium tumefaciens* homologs, respectively. The *A. tumefaciens* homolog of VirB10 has been previously shown to be

an inner membrane protein that spans the periplasm and interacts with other TFSS components to form a functional secretion channel (9). It is proposed for *A. tumefaciens* that VirB10 spans the periplasm in an oligomeric state and stabilizes interactions with other VirB proteins (4, 9, 34). In the absence of VirB10, substrates were not secreted, suggesting that the secretion channel was not formed, which underscores its role in the functionality of the system (6). From computer prediction analysis, it is believed that the *C. jejuni* VirB10 is structurally similar to the *A. tumefaciens* VirB10 and is localized and functions similarly. Mutational analyses of the genes in the ComB system of *H. pylori*, which share homology with the pVir TFSS, resulted in severe reductions in natural competence, suggesting that these ComB proteins form a TFSS that is involved in DNA uptake (15, 16). In *C. jejuni* 81-176, mutation of *virB10* resulted in a modest effect on natural competence and a lesser effect on intestinal cell invasion (2, 3, 35). The *pgl* mutants were previously shown to have a decreased capacity to adhere and invade INT407 cells (28), and in this study we have demonstrated that *pglB* and *pglE* mutants are severely reduced in natural competence, likely due, to a limited degree, to lack of VirB10 glycosylation. The greater competence defect exhibited in the *pgl* mutants compared to that of the *virB10* mutant suggests that additional glycoproteins are required for other steps in natural transformation. This notion is also consistent with the recent description of a putative type II secretion system involved in natural competence in *C. jejuni* (35). It is interesting that 8 of the 10 proteins described by Wiesner et al. (35) contain putative N-linked glycosylation sites by computer prediction. It remains to be determined if any of these proteins are glycosylated and if their function will be affected in the absence of glycosylation. Additionally, the observation that *C. jejuni* strains that lack pVir are competent also reinforces the notion that the pVir TFSS, while modulating competence levels, does not function as the primary DNA uptake system. Nevertheless, the modest effect of *virB10* mutation on natural competence has been demonstrated by two independent groups and was able to be complemented in *trans*, suggesting the defect is genuine (2, 35, and this study). Speculatively, mutation of *virB10* may have an indirect effect on natural competence by destabilizing other proteins that exist in the periplasm or membrane in the absence of a functional TFSS channel.

The identification of a TFSS structural protein that is glycosylated is significant on a number of levels. This is the first example, to our knowledge, of glycosylation of any TFSS protein, as well as the first function ascribed to an N-linked glycan in *C. jejuni*. Secondly, the plasmid-encoded pVir TFSS was presumably acquired through horizontal transfer from an unknown donor. Interestingly, the closest homolog of *C. jejuni* VirB10 is found in *W. succinogenes*, which is also the only other bacterium known to contain a putative N-linked glycosylation system homologous to the *C. jejuni pgl* system (1). Although the biochemical advantage of this general protein glycosylation system remains unknown, it would appear that the gene products of horizontally acquired DNA may be subject to functional restraints from the *pgl* glycosylation system and may need to be further modified to acclimate them to life within the *C. jejuni* host.

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